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(54) Title: A LIVING CHIMERIC SKIN REPLACEMENT

(57) Abstract

The invention comprises skin replacements for improved wound healing. In one embodiment, the skin replacement is chimeric and comprises harvesting autologous epithelial cells from a patient and seeding them onto a biocompatible substrate containing allogeneic epithelial cells cultured *in vitro*. The living chimeric skin replacement may be implanted over a permanent dermal replacement previously implanted at the wound site. In another embodiment the skin replacement is a composite comprising an inner, middle and outer component, comprising (a) an inner component comprising a biocompatible dermal construct having a biodegradable or removable scaffold as a base; (b) a middle component comprising epithelial cells; and (c) an outer component comprising epithelial cells cultured *in vitro* on a dermal construct comprising a dermal portion having a biodegradable or removable scaffold as a base, said dermal portion being combined with a transitional covering and facing inward toward said middle component of epithelial cells. The invention further comprises methods of making and implanting the various skin replacements of the invention.

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A LIVING CHIMERIC SKIN REPLACEMENT

The present application claims the benefit under 35 U.S.C. § 119(e) of United States Provisional Application No. 60/075,704, filed February 24, 1998 which is incorporated by reference herein it its entirety.

1. INTRODUCTION

The present invention relates to a living chimeric skin replacement. This chimeric skin promotes improved wound healing. More specifically, the compositions and methods of this invention effect wound closure by providing a permanent replacement skin containing allogeneic epithelial cells cultured *in vitro* as well as freshly harvested autologous epithelial cells available for immediate implantation. Thus, the invention provides an immediate supply of cells for full coverage of a wound and includes the patients' own keratinocytes.

2. BACKGROUND OF THE INVENTION

Extensive skin wounds due to lacerations, burns, punctures, and skin ulcers often require clinical intervention for healing to occur. Conventional treatments for such injuries often provide less than satisfactory results. Further, approximately 10,000 people in the United States die every year just from skin wounds due to severe burns. Life-threatening burns occur in significant numbers in every form of military engagement and at every level of service. A critical need exists to improve survival rates and the standard of care for such patients suffering from extensive skin wounds such as burns for example and to reduce costs of treatment.

The skin is comprised of both epidermis and dermis tissue. The epidermis contains both living and nonliving layers. The basal layer and the spinous layers are sometimes called the Malpighian layer. Its function is to generate the nonliving outermost barrier layer, the stratum corneum. The epidermis grows from its deepest basal layer or stratum basale. Interspersed between this single layer of columnar-type are the predominant keratinocytes (keratin-producing cells), the pigment-producing cells called melanocytes and Langerhans cells which are derived from bone marrow and play an important role in the immune function of the skin. Cell proliferation occurs in the basal layer and in the lower levels of the stratum spinosum (or spinous layer), where cells are continually pushed toward the surface.

As epidermal cells move toward the surface, they become thinner, dehydrated, hardened and keratinized (a process called differentiation). The spinous layer

actually consists of several layers of cells. Cells move from the stratum spinosum to the stratum granulosum, or the granular layer, which is generally 2-3 cells thick. With time, cells migrate from the stratum granulosum into the stratum lucidum (the transitional cell level), which is the zone of keratinization, where the membrane structure is stabilized by the formation of disulfide cross-links between cysteine residues. Fully formed, flattened, hardened epidermal cells enter the stratum corneum.

The dermis is primarily a supportive connective tissue comprising fibrous tissue containing blood vessels and branches of nerves. The dermis contains fibroblasts, which produce connective tissue elements such as the extracellular matrix proteins, e.g., collagens, fibronectin and elastin. These matrix proteins contribute to the strength and flexibility of the skin. The basement membrane serves, in part, to attach the epidermis to the dermis. The skin also contains accessory organs such as hair follicles and sweat glands. Depending on the severity of a wound, any or all of these elements of the skin can be damaged or lost.

Currently, surgical excision of a burn wound and application of an autograft taken from the patient's unburned skin is the routine method to treat patients with extensive burn injuries in the United States. To provide adequate coverage and to avoid delay, techniques such as mesh grafting are used. This technique involves cutting slits into the skin graft thereby allowing the skin to be spread to cover large wound areas to be grafted. Moreover, the limited availability of healthy skin often requires reharvesting at donor sites which often results in complications such as blood loss, as well as an opportunity for infection and a delay in healing. Thus, in patients with massive burn injury, this poses a significant problem because months may pass before donor sites sufficiently heal to permit reharvesting to produce an adequate number of autografts to accomplish complete wound closure. Consequently, to expedite the process, the autograft skin is usually meshed and stretched so that it can cover large wound areas. Such meshing and stretching results in extensive and undesirable mesh patterns of the healed skin, as well as permanent scarring. Reconstructive surgery may also be required over a period of many years.

Alternatively, when only small areas of unburned autograft skin are available for wound coverage, human cadaveric skin allograft, either cryopreserved or fresh, is currently the standard biologic dressing for coverage of extensive excised burn wounds (Atnip and Burke, 1983, Curr Prob. Surg. 20:623-86; Pruitt and Levine, 1984, Arch. Surg. 119:312-22; Hansbrough, 1987, In: Boswick J, ed. The Art and Science of Burn Care. Rockville, Maryland: Aspen Publ. Inc., 57-63).

While fresh cadaveric skin allograft is the superior covering for excised wounds when autograft skin is not available, fresh skin is in limited supply. Another

problem with cadaveric allograft skin is that it elicits host immunological rejection within several weeks of placement, although survival may be prolonged in the severely burned patient who become markedly immune suppressed secondary to the injury (Ninnemann et al., 1978, Transplantation 25:69-72). Rejection of allograft skin would necessitate its repeat application; rejection may also be accompanied by bacterial colonization and infection of the wounds which can limit subsequent engraftment of both allogeneic and autologous skin. Additionally, subsequent allograft applications frequently undergo accelerated rejection, as a result of previous sensitization of the recipient's immune system. Care of patients with extensive burns thus becomes a race against time to achieve adequate graft coverage before life-threatening infections and other complications occur.

The clinical use of cultured allografts has been attempted repeatedly since their use was reported in 1983. Hefton *et al.*, Lancet 11:428 (1983). Initially, some authors claimed long-term survival and immunological tolerance of these cultured allografts. However, these studies were performed on partial-thickness wounds and the possibility of epithelization from remaining dermal elements, such as hair follicles, could not be excluded. Recent studies suggest that cultured keratinocyte allografts do not survive permanently. Phillips, T.J., Transplantation 44:106 (1987). HLA class I analysis (Gielen *et al.*, Dermatologica 175:166 (1987)), DNA fingerprinting (De Luca *et al.*, Burns 15:303 (1989)), and Y-chromosome analysis (Burt *et al.*, Br. Med. J. 298:915 (1989); Brain *et al.*, Br. Med. J. 298:917 (1989)) have evidenced ultimate replacement of the allograft by recipient keratinocytes.

An alternative approach to increase the availability of graftable tissue has been to obtain a section of undamaged skin from the patient and allow the epithelial cells present in the biopsy to grow in an *in vitro* tissue culture. For example, Green and Rheinwald, U.S. Patent No. 4,016,036 is directed to the serial culture of human epidermal cells such as keratinocytes where primary cultures of keratinocytes are initiated with a single-cell suspension derived from a full-thickness biopsy of a patient's skin, which is minced and treated with trypsin to disaggregate the cells. The cell suspension is plated in culture dishes containing culture medium with various growth factors. The cell suspension overlies a layer of murine fibroblasts that have been irradiated to prevent multiplication. To further expand the cultured keratinocytes, these cultures are treated with trypsin and the disaggregated cells are passed to multiple new dishes, initiating secondary cultures. As they proliferate, the keratinocyte colonies continue to expand until adjacent colonies merge to form a confluent sheet of keratinocytes.

In Green and Kehinde, U.S. Patent No. 4,304,866 further disclosure includes detaching the cultured autologous keratinocytes from the culture dish. To detach them from

the dish, the cultures are treated with Dispase, a protease that digests those proteins involved in cell adhesion to the plastic dish while sparing those involved in cell-cell attachment. Unfortunately, a major drawback is that it takes about three to four weeks to produce a sheet of autologous cells suitable for grafting, creating a significant delay in healing.

Also, the long term success rates of such autologous cultures have been reported to be often less than 50%. One factor which may result in a low success rate is the fact that the cells are enzymatically removed from the culture dish using a neutral protease (commonly dispase) which damages cell surface adhesion receptors, or integrins believed to be important in adherence to the wound. Rennekanpff *et al.*, J. Surgical Res. 62:228-295 (1996).

Pittelkuo and Scott, Mayo Clin. Proc. 61:771-777 (1986) discloses a two phase system for autografting patients with extensive burns. In Pittelkuo, the first phase involved harvesting and culturing a small sample of skin in a serum-free medium for keratinocyte culture. Phase two involves inducing differentiation so that the keratinocytes cells were expanded and induced to form sheets of differentiated keratinocytes for grafting. This process took approximately three weeks. Further, the cells were not cultured on a membrane and the epithelial sheet had to be enzymatically removed with Dispase, and then trypsinized, prior to grafting to the burn patient.

Eisinger, U.S. Patent No. 4,299,819 was one of the first to describe a process for treating burn victims where epidermis is separated from the dermis, dissociating the epidermis into epidermal cells and growing the epidermal cells in the absence of dermal components in a tissue culture medium having a pH of about 5.6 to 5.9. The epidermal sheet is applied to an afflicted area on the burn victim.

Gallico et al., New England Journal of Medicine 311(7):448-451 (1984) describe grafting of skin replacements using autologous epithelial sheets obtained by culturing small skin-biopsy samples. This procedure involves multiple operations where the burn victims first received human-cadaver skin allografts or collagen-glycosaminoglycan-silatic sheets while the autologous cells grow *in vitro*. The autologous skin required several weeks of growth, the sheets of autologous cells further required release of the cultured sheets from culture flasks with Dispase prior to implantation. Further, the allografts needed to be removed prior to implantation of the autologous cultures. Problems associated with this technique include decrease in body temperature and blood loss from allograft removal.

Bell, U.S. Patent No. 4,485,096, describes skin grafts in the rat model containing fibroblasts in collagen lattices and seeded with epidermal cells previously

biopsied from the host recipient. The grafts are cultured *in vitro* and implanted at the wound site.

Various factors can account for the low success rate of skin grafts. For example, the enzymatic treatment used to remove a newly formed epithelial sheet from a tissue culture plate can alter or destroy cell surface molecules, which, in turn, can alter the ability of the cultured cells to adhere to each other or to the wound. In addition, the multilayered cultured epidermal cells can change from a proliferating state into a differentiated state. However, associated with differentiation of the cells is a loss of expression of certain cell adhesion molecules, which, as indicated above, can alter the binding of the cells to each other and to the wound and, therefore, result in a failure to effect wound closure.

While clinical and research experience has demonstrated the poor tolerance of pure allogeneic epithelial grafts in deep thickness burns (Phillips *et al.*, 1991, Transplantation, 51:937), this is primarily the result of the host's immunological response to foreign tissue. Langerhans cells and endothelial cells present in skin have been shown to be the targets of the host's immune response. However, the immune response is generally not immediate and may be significantly delayed in severe wounds, such as burn wounds, where an immunological response raised against the Langerhans and other cells may be compromised due to the damaged tissue surrounding the wound itself.

Various attempts at chimeric skin grafting have been attempted. Auger *et al.*, WO 94/17179 and United States Patent No. 5,610,007, disclose generating a chimeric epithelium *in vitro* where 50:50 and 25:75 mixtures of Balb/c and C3H/HeN mouse keratinocytes were co-cultured to confluence and then enzymatically removed with Dispase (Sigma) to release the cells from the flask's surface. The scientists estimate that their chimeric grafts can be produced in about half the time required for conventional graftable cell sheets of 3-5 weeks. Similarly, Suzuki *et al.*, Transplantation 59(9):1236-1241 (1995), co-cultured C3H/He and BalB/c mouse keratinocytes to examine the graftability of syngeneic (autologous) and allogeneic mouse keratinocytes. Again both the allogeneic and autogenic cells were co-cultured *in vitro* and were enzymatically removed from the culture dish with dispase.

A mixture of allogeneic and autologous microskin grafts (10:1 expansion ratio) were transplanted and overlain with BIOBRANE, onto full thickness skin wounds in the rabbit model. Lin *et al.*, Burns 20(1):30-35 (1994). In Stark and Kaiser, Burns 20(1):534-538 (1994) autologous keratinocytes cultured *in vitro* from skin biopsies of patients with deep partial and full skin thickness burns were grafted as non-confluent single cells suspended in fibrin glue 17 and 25 days after injury. In some of the wounds, the cell-

fibrin suspension was used to attach an additional glycerolized allogeneic split thickness skin graft. Such culturing of the autologous cells *in vitro* prior to grafting creates a delay in grafting and thus, a delay in wound healing. Most of these methods require removal of the cultured cells by enzymatic treatment which has deleterious effect on the cell surface molecules required for cell attachment. This result further detracts from their appeal as desirable skin grafts. Finally, United States Patent 5,693,332 issued to Hansbrough, discloses a method of shortening the time necessary to culture autologous keratinocytes for implantation by growing the keratinocytes on a hydrophilic membrane for one to about 7 days and then contacting the keratinocyte-containing membrane with a wound.

In view of the foregoing problems in the art, there is a need for a superior alternative skin replacement for the treatment of skin-wounded patients. Ideally, such a substitute skin should adhere to the wound rapidly and reproducibly, persist long-term without rejection, and be readily available in large amounts.

3. SUMMARY OF THE INVENTION

The present invention relates to a living chimeric skin replacement, methods for preparing it, and methods of using it, in the treatment of patients with skin wounds caused by a variety of conditions, including for example, lacerations, ulcers and burns. The invention also includes a novel composite skin replacement for use in the treatment of wounds.

The invention is based, in part, on Applicants' discovery that an improved wound covering can be made by grafting allogeneic epithelial cells, such as keratinocytes, which have been cultured on a membrane or other biocompatible substrate *in vitro* and, at or about the time of grafting, adding a relatively small amount of autologous epithelial cells to the allogeneic cell-substrate construct so that the autologous epithelial cells are implanted simultaneously with the allogeneic cells. The autologous cells are preferably keratinocytes which are seeded onto the cultured allogeneic cells just prior to the grafting. Alternatively, the autologous cells can be seeded directly into the wound site, preferably on top of a dermal replacement construct, and the allogeneic cell-substrate construct placed, allogeneic cells facing into the wound, on top of the autologous cells.

In one embodiment, the allogeneic cells are cultured on a membrane or other biocompatible substrate *in vitro* and cryopreserved so that a fresh supply of the graft is readily available to the surgeon. In a preferred embodiment, the allogeneic cells are cultured on a biocompatible, biodegradable membrane which is reversibly hydratable.

In another preferred embodiment, the autologous cells are added to the allogeneic graft at a density of about 1×10^4 cells/cm^2 of graft material.

In another preferred embodiment, the chimeric graft described supra is used in combination with a dermal equivalent such as DERMAGRAFT (Advanced Tissue Sciences, Inc., La Jolla, CA) in deep or full thickness wounds. DERMAGRAFT is a living dermal replacement comprised of a three-dimensional living stromal tissue comprising stromal cells and connective tissue proteins naturally secreted by the stromal cells attached to, and substantially enveloping a biocompatible framework or scaffolding. DERMAGRAFT is described in detail in United States Patent No. 4,963,489, the disclosure of which is incorporated by reference herein in its entirety.

In another preferred embodiment, a composite skin replacement is utilized according to the methods of this invention, wherein a biocompatible dermal construct with a biodegradable or removable scaffolding as a base, such as DERMAGRAFT, is placed into a wound bed, autologous cells from the patient are added on top of the dermal construct, e.g., immediately or after the dermal construct has had time to vascularize, and then an outer component comprising allogeneic epithelial cells cultured on a dermal component comprising a biocompatible dermal construct with a biodegradable or removable scaffolding as a base, combined with a transitional covering such as a BIOBRANE membrane, is placed on top of the autologous cells. Further embodiments of this composite skin replacement include the use of allogeneic cells or autologous cells, or combinations of both, in the middle or outer components of the composite.

In yet another embodiment, allogeneic and autologous epithelial cells can be simultaneously inoculated onto a substrate, cultured for a sufficient time to allow for attachment of the cells to the substrate, and then implanted into a wound site, the cells facing into the wound.

4. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a living chimeric skin replacement. The skin replacement of the invention is a permanent replacement comprising a chimeric construct containing allogeneic and autologous epithelial cells. The allogeneic epithelial cells are cultured on a substrate such as, for example, a biocompatible membrane, dermal construct, glue or gel *in vitro* and, in most cases, cryopreserved. The allogeneic cell-substrate construct is then thawed (if cryopreserved) and placed at the wound site, together with autologous epithelial cells such as keratinocytes, mechanically disrupted skin or microskin bits, the autologous cells having been biopsied, preferably just prior to grafting. If desired, the epithelial replacement may be grafted with a permanent dermal replacement, if the wound is deep.

The present invention provides for an improved skin replacement in that an immediate skin covering of allogeneic cells cultured *in vitro* on a substrate such as a membrane or a dermal construct, is provided to the wound site together with healthy autologous epithelial cells, thereby expediting healing. The ratio of autologous cells to allogeneic cells can be in the range of 1:5 to 1:50. The allogeneic cells provide sufficient coverage before an immunologic response can be raised at the wound site. However, the autologous epithelial cells have a physiological and immunological selective advantage at the wound and eventually replace the allogeneic cells. The amount of autologous cells provided is such that the wound heals with a sufficient coverage of autologous cells before a significant immunologic response occurs. Thus, the present invention has the advantage of being an immediate and available skin graft which, because of the added autologous cells, is a permanent graft without significant immunological rejection.

In one embodiment of the invention, allograft cells from cadaveric skin are inoculated with host autologous cells, and implanted at the wound site. The autologous cells then proliferate and grow progressively replacing the allograft tissue as the host's immunological response is raised against the cadaveric tissue.

The allogeneic epithelial cells are cultured *in vitro* so that they are available for immediate grafting when necessary. The allogeneic epithelial cells are grown on a biocompatible, preferably biodegradable substrate.

By way of example, and not by way of limitation, a living chimeric skin replacement may be produced as follows:

(a) allogeneic epithelial cells are plated onto a biodegradable, biocompatible substrate and are allowed to grow to about 25-90% confluence;

(b) the allogeneic cells and substrate are cryopreserved and stored until needed by a surgeon;

(c) the cells are thawed just prior to grafting;

(d) autologous epithelial cells are harvested and inoculated onto the allogeneic cells at a density of about 1 x 10^4/cm and then implanted at the wound site; and

(e) the chimeric skin graft is covered with a dressing sufficient to adequately immobilize the graft, and protect it from mechanical and infectious forces.

It should be noted that steps (b) and (c) of this method of the invention are optional.

In the case of extensive deep or full thickness wounds, a preferred embodiment of the invention additionally provides that a dermal replacement be placed into the wound site with the subsequent application of the chimeric skin graft. On occasion, depending on the circumstances, it may even be desirous to culture the chimeric graft on the

dermal replacement for several days, prior to grafting to the wound site. The use of a dermal replacement in deep or full thickness wounds helps to create an optional environment for the keratinocytes to attach and proliferate. A major problem for epithelial replacement is the availability of an adequate dermal foundation for grafting at the site of extensive wounds. It is optimal if dermal matrices are readily available. Thus, in a preferred embodiment of the invention, deep or full thickness skin wounds use a cultured dermal replacement such as DERMAGRAFT (Advanced Tissue Sciences, Inc., La Jolla, CA) to support keratinocyte attachment and proliferation. For example, DERMAGRAFT is composed of allogeneic neonatal fibroblasts cultured on biodegradable polyglactin and is a permanent dermal replacement (since the polyglactin is biodegradable). No significant immune response is raised to this graft since there is little immunogenicity associated with these fibroblasts. For example, and not by way of limitation, a three-dimensional chimeric skin graft may be produced in deep or full thickness wounds as follows:

(a) allogeneic epithelial cells are plated onto a biodegradable, biocompatible substrate and are allowed to grow to about 25-90% confluence;

(b) the allogeneic cells and substrate are cryopreserved and stored until needed by a surgeon;

(c) a three-dimensional dermal equivalent is obtained and placed in the wound site;

(d) the allogeneic cells on the biocompatible substrate are thawed as described above;

(e) autologous epithelial cells are harvested and inoculated onto the allogeneic cells;

(f) the chimeric skin graft is attached to the wound by overlaying the graft onto the dermal equivalent; and

(g) the chimeric skin graft is covered with a dressing sufficient to adequately immobilize the graft, and protect it from mechanical and infectious forces.

According to another preferred embodiment of the invention, a chimeric skin replacement is utilized comprising three components: (1) an inner component comprised of a biocompatible dermal construct with a biodegradable or removable scaffolding as a base; (2) a middle component comprised of autologous epithelial cells, such as keratinocytes or mechanically disrupted skin, derived from the patient or an autologous donor, and (3) an outer component comprising allogeneic epithelial cells, such as keratinocytes, cultured on a dermal construct comprising a biocompatible dermal portion with a biodegradable or removable scaffolding as a base combined with a transitional covering such as a BIOBRANE membrane.

According to a preferred embodiment of the invention, the allogeneic cells are grown on and attach to the dermal portion of the outer component (as opposed to the membrane covering) and the dermal side of the outer component containing the cells is facing inward toward the autologous cells of the middle component.

This composite skin replacement can be formed in vitro for use subsequently for treating wounds, such as burns. Alternatively, this composite can be formed at the time of implantation, i.e., the inner dermal construct component can be placed into the wound site, the autologous cells added on top of the dermal component, either immediately or after the dermal component has had time to vascularize, and then the outer component with the dermal side facing inward and containing allogeneic cells is placed on top of the autologous cells. Thus, going from the outermost layer of the composite skin replacement toward the inner most layer, the outer most layer will be the membrane of the outer component, followed by the dermal portion of that component containing the allogeneic cells, followed by the autologous middle layer, followed by the inner most dermal replacement layer.

According to still further embodiments of this composite skin replacement, the middle component can consist of cells from allogeneic sources or a combination of autologous and allogeneic sources. In addition, the cells of this middle component can be in the form of single cell suspensions, microskin bits, disrupted or dispersed skin or in sheet form. Moreover, according to other embodiments of the invention, the cells cultured on the outer component may be autologous or a combination of autologous and allogeneic cells and they may be cultured with or without autologous or allogeneic proteins or combinations thereof.

According to a preferred embodiment of this composite skin replacement, the outer component is a modified version of DERMAGRAFT-TC; also known as TRANSCYTE, DERMAGRAFT-TC is a dermal replacement construct comprising a non-viable dermal component attached to a sialastic membrane such as BIOBRANE (as disclosed in United States Patent No. 5,460,939, the disclosure of which is incorporated herein by reference). According to this invention, a modified version of TRANSCYTE comprises this construct with cellular or protein additions. More particularly, a preferred embodiment is this dermal replacement construct with allogeneic epithelial cells growing on it, with or without added protein factors, e.g., to aid in growth and proliferation.

This outer component serves several purposes. It provides a good barrier to control moisture loss and prevent infection. It also holds all of the other components in place, allowing them the best opportunity to adhere to the wound site in an environment that optimizes their growth. Furthermore, it is translucent and thus allows for visual observation during the healing process.

Both the inner component and the outer component can be grown or formed in vitro and cryopreserved and stored for subsequent application in vivo. Thus, according to a preferred embodiment of the invention, a non-viable DERMAGRAFT is placed into a wound bed with mechanically dispersed or disrupted autologous skin from the patient placed on top and covered with a piece of TRANSCYTE that has been modified by culturing allogeneic keratinocytes on one aspect, preferably on the side facing into the wound, and/or coating with proteinaceous solutions. The patient's skin cells grow to confluence atop the inner DERMAGRAFT and then the outer component is eventually removed or may slough off.

The invention is discussed in more detail in the subsections below, solely for purposes of description and not by way of limitation. For clarity of discussion, the specific procedures and methods are described herein. These techniques are merely illustrative for the practice of the invention. Analogous procedures and techniques are equally applicable.

4.1. SUBSTRATES FOR CULTURING THE ALLOGENEIC CELLS *IN VITRO*

The substrate should be biocompatible and preferably biodegradable so that the substrate degrades over time and does not need to be removed at a later date. Also, the substrate, such as a membrane substrate for example, should be one which is reversibly hydratable, so that it allows for absorption and/or release of exudate in order to prevent the accumulation of exudate at the wound site, and prevents desiccation of the wound. The membrane preferably has a small pore size so that it reduces or inhibits the ability of bacteria to infect the wound. The substrate should readily adhere to the wound or be capable of being treated with a bioadhesive so that adherence to the wound occurs without harming the epithelial cells or significantly changing the moisture vapor transmission (MVTR) of the membrane. The substrate should also be adequately malleable so that it conforms to variable wound surfaces.

In one embodiment, the allogeneic epithelial cells are grown on a synthetic hydrophilic polyurethane membrane dressing, such as, for example, HYDRODERM (Wilshire Medical, Inc., Dallas, TX) or SPYROFILM (Polymedica Inc., Denver, CO). Both films have high but variable water vapor permeability and a biocompatible adhesive coating which covers a portion of the membrane surface. When they become wet, these polyurethane membranes significantly increase their water-vapor permeability. The MVTR of HYDRODERM is a function of the degree of wetness of the membrane. Normal skin has a range of MVTR of 200-2000 $g\text{-}m^{-2}\text{-}d^{-1}$. Damaged or burned skin may have moisture transmission as high as 3000-5000 $g\text{-}m^{-2}\text{-}d^{-1}$. Conventional hydrophobic polyurethane films with equilibrium water contents of <1% have moisture vapor transmissions (MVTRs) of

1500-2000 g-m^{-2}-d^{-1}. However, when coated with a conventional medical adhesive, the MVTR falls to 500-1000 g-m^{-2}-d^{-1} because the adhesive blocks water vapor transmission. The MVTR should not be one that allows for maceration of tissues, channeling, and leakage and potentiates infections.

Another possible hydrophilic membrane is the outer layer film of "MITRAFLEX" (PolyMedica Industries, Inc.; Golden CO) which is a flexible, transparent polyurethane membrane that allows moisture vapor to be transmitted but is impermeable to other liquids and to microorganisms (see Reed, J. Biomat. Appl. 6:26-31 (1990)).

The cultured allogeneic epithelial cells may be inversely applied to the wound without the necessity of enzymatically detaching the cells from the membrane, for example. Keratinocytes cultured on these membranes have been shown to highly express integrins involved in the wound healing processes.

Hyaluronic acid membranes may also be used for culturing the keratinocytes. Hyaluronic acid has been shown to have the properties of biocompatibility and low antigenicity. Cortivo *et al.*, Biomaterials 12:727 (1991). Partial to complete esterification of hyaluronic acid with ethyl or benzyl linkages produces materials of high biocompatibility and intermediate to long half-lives. For example, LASERSKIN, Fidia Advanced Biocopolymers, Abano Terme, Italy can function as a carrier system for the cultured allogeneic keratinocytes of the invention. This 200-μm-thick membrane has microholes of 40-60 μm in diameter which allow vapor permeation and also migration of cells.

Another embodiment of the invention is the use of fibronectin mats. Ejim *et al.* reported the use of fibronectin mats as an *in vitro* support for keratinocytes (Ejim *et al.*, Biomaterials 14:742 (1993)). Furthermore, it was shown that fibronectin inhibits differentiation of cultured keratinocytes. Adams *et al.*, Cell 63:425 (1990). Keratinocytes cultured on fibronectin mats showed strong expression of integrins on keratinocytes of the epithelium in healing wounds such as integrin $\alpha 5\beta 1$ and increasing $\alpha v\beta 5$ expression. Prajapati *et al.*, 5th Annual Meeting of the European Tissue Repair Society, Padova, Italy, August/September 1995.

Another substrate for culturing the allogeneic cells is a dermal construct comprising a biocompatible dermal portion with a biodegradable or removable scaffolding as a base combined with a transitional covering such as a BIOBRANE membrane. The allogeneic cells can be applied to either aspect or side of the dermal construct, but preferably are applied to the dermal aspect. The allogeneic cells can be cultured with or without additional protein factors applied to the dermal construct.

Another substrate for culturing the allogenic epithelial cells of the invention is fibrin glue or a collagen gel. Any membrane or substrate capable of supporting the

growth of cultured epithelial cells may be used in the invention. Preferably, the cells, once cultured on the membrane or substrate, do not have to be enzymatically removed.

When culturing the cells on a substrate such as a membrane, the membrane should be kept taut, such as by attachment to some type of a framework to prevent wrinkling or shrinkage of the membrane when the cells are growing on the membrane.

Still another substrate that can be used to culture the allogeneic cells is a hydrogel, which is a polymer composition, preferably prepared from hydrophilic polymers, wherein the polymer, natural or synthetic, is cross-linked, e.g., via photopolymerization, to create a three-dimensional lattice structure which traps water molecules to form a gel. Polymers useful for forming hydrogels include polysaccharides or carbohydrates, such as alginates, polyphosphazines, polyethylene glycol or polyethylene oxide. Preferably, the hydrogels used herein are polymerized prior to the addition of the allogeneic cells. Details for preparing hydrogels for use in this invention are set forth in the United States application Serial No. 08/862,740, filed May 23, 1997, the disclosure of which is incorporated herein by reference in its entirety.

For an overview of various substrates suitable for culturing keratinocytes for implantation at a wound site, see Rennekampf *et al.*, J. Surgical Research 62:288-295 (1996).

4.2. ESTABLISHMENT OF THE ALLOGENEIC CELLS ON THE SUBSTRATE

Allogeneic epithelial cells may be cultured on the membranes and substrates of the invention by any appropriate means known in the art, such as, for example, the methods contained in Doyle, A., Griffiths, J.B., and Newell, D.G., "Cell & Tissue Culture: Laboratory Procedures," Wiley Publishers (1995).

For example, and not by way of limitation, keratinocytes and melanocytes may be isolated as follows. A tissue sample, e.g. foreskin, may be trimmed so that the entire surface may be easily exposed to antibiotics. For example, subcutaneous tissue is removed with sterile scissors and approximately 10 ml PBS with Ca++, Mg++ is added to the dish to rinse off cellular debris. The tissue is then washed in a concentrated antibiotic solution and let sit for 5 minutes. The tissue is then transferred to 0.25% trypsin/EDTA, cut lengthwise into strips or squares approximately 3 mm wide with sterile scalpel or scissors. The cut tissue in trypsin is placed in a CO2 incubator at 37 ^{0}C, 5% C02, 90% humidity for 2.5-3 hrs. The tissue pieces may be removed from the trypsin solution, and the epidermis separated from the dermis using curved forceps. The epidermis may be placed in a conical tube, and about 0.15% trypsin in calcium-magnesium free PBS, may be used to digest the tissue into a single cell suspension; to facilitate this process, the sample may be repeatedly

aspirated into and out of a Pasteur pipette. When the sample appears to be a single cell suspension, it may be centrifuged at 300 x g for about 10 minutes and the cell pellet resuspended by gently tapping the bottom of the centrifuge tube. Approximately 1 ml Keratinocyte Complete Culture Medium (KCCM) is added to the cells and the tube is gently tapped to further suspend the pellet. 5 ml KCCM is then added and the cell suspension pipetted up and down several times until the solution is homogenous. The cells are then transferred into culture vessels, e.g., T150 flasks, and the flasks rotated for even distribution of cells. The flasks are placed in a CO2 incubator at 37 ^{0}C, 5% C02, 90% humidity and the cells are mitotically expanded.

These allogeneic epithelial cells are then applied to a substrate, e.g., a membrane in a petri dish, and allowed to grow, e.g., at 37 ^{0}C, 5% C02, 90% humidity. Once an appropriate stage of confluence is reached, preferably about 25-90% confluence, more preferably, about 70-80% confluence, the cells are either cryopreserved or used for implantation. The cultured epithelial sheets may be cryopreserved by methods known in the art. See, for example, the methods described in Tubo *et al.*, U.S. Patent No. 5,145,770 which is incorporated by reference. The allogeneic cells on the substrate are then thawed at the appropriate time, generally just prior to grafting.

In an alternative embodiment of this invention, the allogeneic cells can be genetically engineered prior to being cultured on the substrate to "knock out" expression of factors that promote patient immunological responses against the implanted allogeneic cells. Negative modulatory techniques for the reduction of target gene expression levels or target gene product activity levels are discussed below. "Negative modulation", as used herein, refers to a reduction in the level and/or activity of target gene product relative to the level and/or activity of the target gene product in the absence of the modulatory treatment. The expression of a gene native to the allogeneic cell can be reduced or knocked out using a number of techniques, for example, expression may be inhibited by inactivating the gene completely (commonly termed "knockout") using standard homologous recombination techniques. Usually, an exon encoding an important region of the protein (or an exon 5' to that region) is interrupted by a positive selectable marker (for example *neo*), preventing the production of normal mRNA from the target gene and resulting in inactivation of the gene. A gene may also be inactivated by creating a deletion in part of a gene, or by deleting the entire gene. By using a construct with two regions of homology to the target gene that are far apart in the genome, the sequences intervening the two regions can be deleted. Mombaerts et al., 1991, Proc. Nat. Acad. Sci. U.S.A. 88:3084-3087.

Antisense and ribozyme molecules which inhibit expression of the target gene can also be used in accordance with the invention to reduce the level of target gene

activity. For example, antisense RNA molecules which inhibit the expression of major histocompatibility gene complexes (HLA) have been shown to be most versatile with respect to immune responses. Furthermore, appropriate ribozyme molecules can be designed as described, e.g., by Haseloff et al., 1988, Nature 334:585-591; Zaug et al., 1984, Science 224:574-578; and Zaug and Cech, 1986, Science 231:470-475. Still further, triple helix molecules can be utilized in reducing the level of target gene activity. These techniques are described in detail by L.G. Davis et al., eds, Basic Methods in Molecular Biology, 2nd ed., Appleton & Lange, Norwalk, Conn. 1994.

Using any of the foregoing techniques, the expression of MHC class II molecules can be modified in the allogeneic cells of this invention in order to reduce the risk of rejection of the chimeric implant.

For example, the histocompatibility of the implanted allogeneic cells can be enhanced or improved if, after undesirable MHC antigens have been knocked out, one or more genes encoding one or more recipient-compatible HLA antigens are transfected into the allogeneic cells prior to implantation. Thus, via known genetic engineering techniques, the antigenicity of the allogeneic cells can be modified to improve histocompatibility of the cells with the recipient patient.

4.3 HARVESTING THE AUTOLOGOUS EPITHELIAL CELLS

Autologous epithelial cells are isolated, preferably immediately prior to transplantation by the following method. Healthy tissue from the autologous donor is washed with 70% Ethanol, cold proviodine, and then 70% Ethanol. The washed tissue is dissected to remove the epidermal layer from the dermal layer. The epidermal layer is then washed in cold Phosphate Buffered Saline and suspended in a 0.25% trypsin-EDTA solution with 2.5 mg/ml dispase and mechanically disrupted for 10 minutes at room temperature. The epithelium pellet is resuspended in PBS at an appropriate concentration. The autologous cells are then added to the cultured allogeneic cells on the surface of the substrate described supra or alternatively, implanted directly into the wound site and then covered with the allogeneic cell-substrate construct which is inverted into the wound, cells facing into the wound as described herein.

4.4. IMPLANTING THE LIVING CHIMERIC SKIN GRAFT

According to one embodiment of the invention, a cultured dermal skin replacement such as DERMAGRAFT is placed into the wound bed. The DERMAGRAFT may be cryopreserved and thawed prior to use, in which case, it is rinsed in NaCl solution (e.g., 0.9% normal saline). The wound site is preferably cleaned/sterilized prior to

implantation by scrubbing or rinsing with a sterilizing solution, e.g., iodine surgical scrub (e.g., 0.75% titratable iodine) or 0.9% sodium chloride solution. After placement of the DERMAGRAFT into the wound (it may be optionally sutured to the site), the allogeneic cells cultured on the biocompatible substrate as described above are inoculated with autologous epithelial cells harvested from the autologous donor as described above. The autologous cells adhere to the surface of the allogeneic cell-substrate construct (i.e., the cells adhere to the surface of the construct containing the allogeneic cells) and the construct is inverted and placed into the wound site, i.e., with the cells facing into the wound and toward the DERMAGRAFT. The wound site is then covered with a protective dressing or may be closed and sealed with sutures.

WHAT IS CLAIMED IS:

1. A living chimeric skin replacement produced by the process comprising:

harvesting autologous epithelial cells from a patient and seeding them onto a biocompatible substrate containing allogeneic epithelial cells cultured *in vitro*.

2. The living chimeric skin replacement of claim 1 wherein the allogeneic cells comprise keratinocytes and/or melanocytes.

3. The living chimeric skin replacement of claim 1 wherein the allogeneic cells are confluent.

4. The living chimeric skin replacement of claim 1 wherein the allogeneic cells are about 25-90% confluent.

5. The living chimeric skin replacement of claim 1 wherein the allogeneic cells are genetically engineered cells.

6. The living chimeric skin replacement of claim 1 wherein the autologous cells comprise keratinocytes and/or melanocytes.

7. The living chimeric skin replacement of claim 1 wherein the substrate is biodegradable.

8. The living chimeric skin replacement of claim 7 wherein the substrate is a synthetic hydrophilic polyurethane membrane, a hyaluronic acid membrane, a fibronectin mat, a fibrin glue, a collagen gel, or a hydrogel.

9. The living chimeric skin replacement of claim 1 wherein the autologous cells are seeded at a density of about $1x10^4/cm^2$.

10. The living chimeric skin replacement of claim 1 wherein the ratio of autologous cells to allogeneic cells is in the range of 1:5 to 1:50.

11. The living chimeric skin replacement of claim 1 wherein the biocompatible substrate containing allogeneic cells cultured *in vitro* has been cryopreserved and thawed prior to seeding with autologous cells.

12. A method for making a chimeric skin replacement comprising :
(a) harvesting autologous epithelial cells from a patient; and
(b) seeding the autologous cells onto a biocompatible substrate containing allogeneic epithelial cells cultured *in vitro*.

13. A method for implanting a chimeric skin replacement at a wound site comprising:
(a) harvesting autologous epithelial cells from a patient;
(b) seeding the autologous cells onto a biocompatible substrate containing allogeneic epithelial cells cultured *in vitro* to form a chimeric skin replacement; and
(c) implanting the living chimeric skin replacement at the wound site by inverting the chimeric skin replacement so that the cells face into the wound site.

14. A method for implanting a chimeric skin replacement at a wound site comprising:
(a) harvesting autologous epithelial cells from a patient;
(b) seeding the autologous epithelial cells into the wound site; and
(c) implanting a biocompatible substrate containing allogeneic epithelial cells cultured *in vitro* into the wound site by inverting the substrate so that the allogeneic cells face inward toward the autologous cells.

15. The method of claim 12, 13 or 14 wherein the allogeneic cells comprise keratinocytes and/or melanocytes.

16. The method of claim 12, 13 or 14 wherein the allogeneic cells are confluent.

17. The method of claim 12, 13 or 14 wherein the allogeneic cells are about 25-90% confluent.

18. The method of claim 12, 13 or 14 wherein the allogeneic cells are genetically engineered cells.

19. The method of claim 12, 13 or 14 wherein the autologous cells comprise keratinocytes and/or melanocytes.

20. The method of claim 12, 13 or 14 wherein the substrate is biodegradable.

21. The method of claim 20 wherein the substrate is a synthetic hydrophilic polyurethane membrane, a hyaluronic acid membrane, a hyaluronic acid membrane, a fibronectin mat, a fibrin glue, a collagen gel or a hydrogel.

22. The method of claim 12, 13, or 14 wherein the autologous cells are seeded at a density of about $1x10^4/cm^2$.

23. The method of claim 12, 13 or 14 wherein the ratio of autologous cells to allogeneic cells is in the range of 1:5 to 1:50.

24. The method of claim 12, 13 or 14 wherein the biocompatible substrate containing the allogeneic cells cultured *in vitro* has been cryopreserved and thawed prior to seeding with autologous cells.

25. The method of claim 13, wherein the harvested autologous cells are seeded at about the time of implantation onto the biocompatible substrate containing the cultured allogeneic epithelial cells.

26. The method of claim 13 wherein the seeded autologous cells are cultured *in vitro* on the biocompatible substrate containing the allogeneic epithelial cells prior to implantation.

27. The method of claim 13 or 14 wherein the wound site is a deep or full thickness wound.

28. The method of claim 27 further comprising implanting a dermal replacement into the wound site prior to implanting the chimeric skin replacement, the

chimeric skin replacement being inserted so that the cells of the chimeric skin replacement face inward toward the dermal replacement.

29. A composite skin replacement, having an inner, middle and outer component, comprising:

(a) an inner component comprising a biocompatible dermal construct having a biodegradable or removable scaffold as a base;

(b) a middle component comprising epithelial cells; and

(c) an outer component comprising epithelial cells cultured *in vitro* on a dermal construct comprising a dermal portion having a biodegradable or removable scaffold as a base, said dermal portion being combined with a transitional covering and facing inward toward said middle component of epithelial cells.

30. The composite skin replacement of claim 29 wherein the dermal construct of the inner component comprises mesenchymal stem cells.

31. The composite skin replacement of claim 29 wherein the epithelial cells of the outer component are cultured *in vitro* on the dermal portion of the construct.

32. The composite skin replacement of claim 29 wherein the transitional covering of the outer component is a membrane.

33. The composite skin replacement of claim 32 wherein the membrane is a sialastic membrane.

34. The composite skin replacement of claim 29 wherein the epithelial cells of the outer component are autologous, allogeneic or a combination of autologous and allogeneic cells.

35. The composite skin replacement of claim 34 wherein the outer component has been further modified by the addition of autologous, allogeneic or a combination of autologous and allogeneic proteins.

36. The composite skin replacement of claim 29 wherein the epithelial cells of the middle component are in the form of sheets, single cell suspensions, microskin bits, or disrupted or dispersed skin.

37. The composite skin replacement of claim 29 wherein the epithelial cells of the middle component are autologous, allogeneic or a combination of autologous and allogeneic cells.

38. The composite skin replacement of claim 29, 34 or 37 wherein the epithelial cells are keratinocytes and/or melanocytes.

39. The composite skin replacement of claim 29, 34 or 37 wherein the epithelial cells are genetically engineered.

40. A method of implanting a composite skin replacement into a wound site, said skin replacement comprising an inner, middle and outer component where: (a) the inner component comprises a biocompatible dermal construct having a biodegradable or removable scaffold as a base; (b) the middle component comprises epithelial cells; and (c) the outer component comprises epithelial cells cultured *in vitro* on a dermal construct comprising a dermal portion, having a biodegradable or removable scaffold as a base, in combination with a transitional covering, the dermal portion facing inward toward said middle component of epithelial cells; and implanting the skin replacement into said wound site so that the inner component (a) is in direct contact with the deepest portion of the wound site and the outer component (c) is closest to the outermost portion of the wound site.

41. A method for making a composite skin replacement *in vivo* at a wound site comprising:

(a) implanting an inner biocompatible first dermal construct having a biodegradable or removable scaffold as a base into the wound site;

(b) harvesting autologous epithelial cells from a patient;

(c) seeding the autologous epithelial cells on top of the inner dermal construct in the wound site; and

(d) implanting, on top of the autologous cells, an outer second dermal construct having epithelial cells cultured *in vitro* and comprising a dermal portion having a biodegradable or removable scaffold as a

base, in combination with a transitional covering, so that the epithelial cells of the outer dermal construct face into the wound site.

42. A method for making a composite skin replacement *in vitro*, comprising:

(a) seeding epithelial cells on a first biocompatible dermal construct having a biodegradable or removable scaffold as a base; and

(b) placing a second dermal construct having epithelial cells cultured thereon and comprising a dermal portion having a biodegradable or removable scaffold as a base, in combination with a transitional covering, onto the first dermal construct, such that the cells of the second dermal construct face the cells on the first dermal construct.

43. The method of claim 41 wherein the epithelial cells of the outer dermal construct are autologous, allogeneic or a combination of autologous and allogeneic cells.

44. The method of claim 42 wherein the epithelial cells of the first dermal construct are autologous, allogeneic or a combination of autologous and allogeneic cells.

45. The method of claim 42 wherein the epithelial cells of the second dermal construct are autologous, allogeneic or a combination of autologous and allogeneic cells.

46. The method of claim 40, 41 or 42 wherein the epithelial cells are keratinocytes and/or melanocytes.

47. The method of claim 40, 41 or 42 wherein the epithelial cells are genetically engineered.

48. The method of claim 41 or 42 wherein the epithelial cells of the second dermal construct are cultured *in vitro* on the dermal portion of the construct.

49. The method of claim 40, 41 or 42 wherein the transitional covering is a membrane.

50. The method of claim 49 wherein the membrane is a sialastic membrane.